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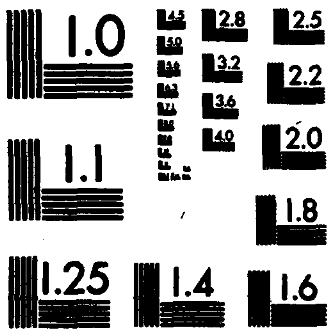


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20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

Immunoenhancing drugs were studied both for their general effects on host immunity and their effects on immune interactions with microbial infections. These studies focused preferentially on defined single molecules with immunomodulating properties, and on fungi as the microbes of interest. The prototype immunomodulator studied was muramyl dipeptide (MDP). We showed that MDP in vivo enhanced cellularity and mitogen responses in some lymphoid compartments (lymph nodes) and depressed responses in others (spleen). The optimum

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dose and interval for effect were defined, and subpopulations of the compartments were fractionated which were responsible for differences observed with different mitogens (including suppressor cells). We also showed that polymorphonuclear leukocytes (PMN) can be activated as a byproduct of an immunological reaction, and in that state have enhanced fungicidal activity and can kill some fungi which normal PMN cannot. Activation could be reproduced with lymphokines. We showed that CP46665, a lipoidal amine, in vivo enhances fungicidal activity of PMN and alveolar macrophages (AM), but had no in vitro effect. However, interferon, produced by gene cloning recombinant DNA techniques, had an adverse effect on PMN or AM antifungal activity. Thymosin was shown to boost T lymphocyte responses of coccidioidomycosis patients to fungal antigen, whereas it depressed antigen responses in a human granulomatous mycobacterial infection, and nonspecific responses were generally unaffected. Thus the specificity of and potential clinical applicability of several immunomodulating agents were demonstrated.

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I. A significant effort has been put in studies on the mechanism of immunomodulation (section 1, beginning p. 7 of our proposal). These studies focused on muramyl dipeptide (MDP) as the prototype immunomodulator, as indicated.

1. The effect of MDP dose on lymph node cellularity and responsiveness to mitogens was studied. Doses of 600 and 1200 mcg were compared to the 200 mcg x 4 days dose (the reference standard, as discussed). These doses were given IP days -4, -3, -2, -1. On day 0, lymph node cells (LNC) were collected, and single cell suspensions prepared and counted. We found a 6-fold increase in MDP dose did not affect the increased yield of LNC after MDP treatment as we reported earlier (2.6 times that in saline-treated controls), nor increase the enchanced response to concanavalin A (con A) or lipopolysaccharide (LPS). The results indicate that for further studies of the mechanism, the 200 mcg dose cannot readily be improved upon in this regimen.

Table 1

Effect of MDP dose on responsiveness of LNC to Con A and LPS (cpm)

| Treatment | NIL | Con A | LPS |
|---|---|---|---|
| Saline | 438 | 58,880 | 2,908 |
| MDP 200 mcg | 2,999 | 128,582 | 13,211 |
| 600 mcg | 3,148 | 115,025 | 11,090 |
| 1200 mcg | 2,896 | 111,705 | 11,453 |

2. The effect of dose on spleen cellularity and responsiveness to mitogens was studied. Regimens as given above were studied. Increasing the dose of MDP up to 6-fold did not increase the number of spleen cells (saline controls, 138 x $10^6$ cells; MDP-treated, 140-168x$10^6$ cells). Our previous finding of suppressed responses to mitogens in the splenic compartment was confirmed. With phytohemagglutinin (PHA), responses were further depressed by increases in MDP dose. Responses to con A appear maximally depressed at the 200 mcg x 4 day regimen. Therefore, further studies of the mechanisms of the suppressed response to mitogens should use the higher dose when studying PHA.

Table 2

Effect of MDP dose on responsiveness of spleen cells to mitogens (cpm)

| Treatment | NIL | Con A | PHA |
|---|---|---|---|
| Saline | 927 | 33,051 | 58,609 |
| MDP 200 mcg | 1,186 | 12,800 | 46,763 |
| 600 mcg | 1,280 | 13,540 | 40,041 |
| 1200 mcg | 782 | 26,189 | 34,393 |

3. The effect of time after MDP on lymph node responses to mitogens was studied. We found the increased celluarity in lymph nodes following MDP treatment (200 mcg x 4) rapidly diminishes, such that cell yield falls from 52 x $10^6$ lymph node cells/mouse on day 0 to near saline control levels on day +4 (27 x $10^6$). The hyper-responsiveness to con A and LPS likewise wane in parallel. Enhanced responses to con A (2.2 x controls) and LPS (4.5 x) on day 0 were decreased on day +4 (con A, 1.4 x; LPS 3.6 x) and day +7 (con A, 1.5 x; LPS, parity). This defines the duration of effect on lymph node cells of this immunomodulator.

Table 3

Effect of time past MDP treatment on LNC responses to mitrogens (cpm)

| Days Post Treatment | | NIL | Con A | LPS |
|---|---|---|---|---|
| 0 | Saline | 438 | 58,880 | 2,908 |
| | MDP | 2,999 | 128,582 | 13,211 |
| 4 | Saline | 440 | 53,741 | 1,195 |
| | MDP | 1,316 | 73,448 | 4,343 |
| 7 | Saline | 961 | 49,662 | 9,268 |
| | MDP | 1,239 | 76,836 | 8,176 |

4. The effect of time after MDP treatment on spleen cell depression was studied. A 200 mcg x 4 regimen was used. Spleen cellularity did not change with time. However, hyporesponsiveness to con A rebounded by day +4 (120% of control), and the PHA response returned to normal. These results demonstrate the transience of the depressed responses to T-cell mitogens by spleen cells after MDP, and that a rebound occurred for con A.

Table 4

Effect of time post MDP treatment on spleen cell responses to mitogens (cpm)

| Days Post Treatment | | NIL | Con A | PHA |
|---|---|---|---|---|
| 0 | Saline | 927 | 33,051 | 58,609 |
| | MDP | 1,186 | 12,800 | 46,763 |
| 4 | Saline | 872 | 56,047 | 22,641 |
| | MDP | 819 | 67,307 | 22,347 |
| 7 | Saline | 896 | 46,844 | 23,328 |
| | MDP | 1,239 | 55,319 | 18,354 |

5. We studied the effect of MDP treatment on the number of T and B cells in lymph nodes and spleens. These experiments tested the possibility that hyper-responsiveness of LNC and hyporesponsiveness of spleen cells after MDP could be accounted for by increased or decreased numbers of T cells. Cells from both compartments after saline or MDP treatment were treated with anti T-cell sera + C′ or anti-Ig +C′, and viable cells enumerated. We found the percent of T cells in lymph nodes from MDP treated mice was unchanged. We also found the % T cells in spleens from MDP treated mice was unchanged. In contrast, the % cells sensitive to anti-Ig + C′(B cells) was increased slightly in lymph node cells and in spleens after MDP treatment. These results indicate the hyper-responsiveness of lymph node cells, and the hypo-responsiveness of spleen cells to T cell mitogens after MDP, couldn't be accounted for by higher or lower % T cells. The slight increased % B cells could contribute to the increased response of lymph node cells to LPS, a point to be addressed in further experiments. The results suggest the regulation of responsiveness of T cells up or down in the 2 compartments results from interaction with other cell types, or in changes in T cell subsets. Further study is indicated.

Table 5

Effect of MDP treatment on the number of T and B cells in lymph nodes and spleens

| | Anti-T + C′ | Anti-Ig + C′ |
|---|---|---|
| Lymph Node | | |
| Saline | 55 ± 8% | 22.5% |
| MDP | 55 ± 2 | 32.5 |
| Spleen | | |
| Saline | 37 ± 0.4 | 34.5 |
| MDP | 38 ± 2.8 | 41.5 |

6. We studied the effect of MDP treatment on the response of lymph node cells nonadherent to nylon wool. These studies extended our studies to pinpoint the cells responsible for enhanced or depressed responses. Lymphocytes nonadherent to nylon wool are almost exclusively (>95%) T cells. Lymph node cells fractionated by nonadherence to nylon wool were tested with T cell mitogens. These T cells from MDP-treated mice were hyperresponsive to con A but not PHA. The results suggest this population of T cells after MDP treatment acquires enhanced responsiveness to con A, or is enriched for a subpopulation of con A responding T cells, and that these cells contribute to the enhanced response of unfractionated lymph node cells. In contrast, the increased PHA response of unfractionated lymph node cells can't be accounted for by the PHA responsiveness of nylon wool nonadherent T cells. This suggests the enhanced PHA responsiveness requires cells adherent to nylon wool.

Table 6.

Effect of MDP treatment on the responses of lymph node cells nonadherent to nylon wool (cpm)

| | Unfractionated | | Nonadherent | |
|---|---|---|---|---|
| Mitogens | Saline | MDP | Saline | MDP |
| None | 358 | 3,013 | 205 | 606 |
| Con A | 42,452 | 172,098 | 14,769 | 44,387 |
| PHA | 23,446 | 42,986 | 18,282 | 19,737 |
| LPS | 1,637 | 9,983 | 386 | 608 |

7. We studied the effect of MDP on spleen cells nonadherent to nylon wool. Spleen cells from saline or MDP treated mice fractionated by nonadherence to nylon wool were tested for their ability to respond to con A, PHA and LPS. Although nylon wool nonadherent spleen cells make poor responses to con A, such cells from MDP treated mice made enhanced (3x) responses compared to cells from saline treated controls. In contrast their response to PHA was depressed, e.g., 2/3 of the response made by control cells. LPS results were like those seen with con A. These results suggest that the depressed responsiveness of spleen cells from MDP treated mice to PHA could be partially due to the hyporesponsiveness of the T cells nonadherent to nylon wool. On the other hand, the depressed responsiveness of spleen cells to con A could not be attributed to splenic T cells nonadherent to nylon wool.

Table 7

Effect of MDP treatment on the responses of spleen cells nonadherent to nylon wool (cpm)

| | Unfractionated | | Nonadherent | |
|---|---|---|---|---|
| Mitogens | Saline | MDP | Saline | MDP |
| None | 973 | 823 | 187 | 363 |
| Con A | 62,258 | 20,566 | 1,458 | 4,414 |
| PHA | 60,914 | 23,317 | 37,107 | 27,638 |
| LPS | 16,266 | 7,467 | 2,242 | 2,849 |

8. One of the methods of detecting suppressor cell activity is to measure the effect of mixing the test population with normal cells. When spleen cells from MDP treated mice were mixed with spleen cells from saline treated mice in various ratios, the mean calculated response to con A, i.e., (cpm of "saline" cells + cpm of "MDP" cells) /2, was depressed, and the effect more potent with more MDP spleen cells present. This was also true for responses to PHA, especially when cultures contained 50% saline cells and 50% MDP cells, where the mean calculated response was depressed by 31% (Table 8). The mean calculated responses to LPS were depressed, but only when cocultures contained 50 to 80% MDP cells (Table 8). These results demonstrate that spleen cells from MDP treated mice contain cells capable of depressing responses of normal spleen cells to T and B cell mitogens.

Table 8

Effect of mixing spleen cells from MDP and saline treated mice on responses to con A, PHA, and LPS

| Ratio of spleen cells | Percent of mean calculated response | | |
|---|---|---|---|
| Saline: MDP[a] | Con A | PHA | LPS |
| 80:20 | 28% | 24% | 0% |
| 50:50 | 48% | 31% | 16% |
| 20:80 | 62% | 14% | 18% |

[a] Spleen cells from MDP treated mice made depressed responses to con A (51%), PHA (17%), and LPS (14%) compared to saline controls.

9. We have evidence that the suppressor cells in spleen cells of MDP-treated animals are T cells. Treatment of such cells with anti-T cells sera + C' abolished the suppressor effect of MDP spleen cells on normal spleen cell responses.

Table 9

Effect of anti-T cell sera + C' on spleen cells from MDP treated animals (cpm)

| Ratio of spleen cells<br>Saline treatment: MDP treatment | Con A | PHA | LPS |
|---|---|---|---|
| 100:0 | 58,000 | 9,000 | 4,000 |
| 50:50 | 25,000(0%)[a] | 4,500(8%) | 1,800(0%) |
| 0:100 | 600 | 537 | 1,500 |

[a] Depression of mean calculated response

10. Mixing experiments were applied to LNC, and the enhancement seen by MDP on these cells. LNC from MDP-treated and from normal animals were mixed in various ratios. We showed that LNC from MDP-treated animals actually enhanced the response of such cell mixtures beyond that expected from the ratio. This suggests that MDP treatment results in a LNC population that enhances the responsiveness of normal LNC.

Table 10

Effect of mixing lymph node cells from MDP and saline treated mice on responses to con A, PHA and LPS (cpm)

| Ratio of LNC<br>Saline treatment: MDP treatment | Con A | PHA | LPS |
|---|---|---|---|
| 100:0 | 59,694 | 15,833 | 632 |
| 80:20 | 124,961(41%)[a] | 25,708(36%) | 1,460(18%) |
| 50:50 | 128,842(27%) | 27,001 (35%) | 2,736(25%) |
| 20:80 | 136,115(15%) | 22,720(0%) | 3,448(16%) |
| 0:100 | 129,797 | 19,421 | 3,477 |

[a] Enhancement of mean calculated response

11. Preliminary experiments were recovery of some nylon wool adherent spleen cells suggest a firmly bound suppressor cell (not easily recoverable) for PHA responsiveness after MDP treatment. Adherent but recoverable spleen cells from MDP animals appear not to be hyporesponsive, consistent with the hypothesis that the suppressor for con A and LPS responses is in the nonadherent population, or is a firmly bound suppressor cell.

Preliminary studies with plastic adherence suggest plastic nonadherent spleen cells from MDP treated mice are hyporesponsive to conA, whereas adherent cells are normal. Moreover, the plastic nonadherent cells appear to contain a suppressor cell.

Further preliminary mixing studies suggest that whereas spleen cells from MDP mice suppress normal spleen cells con A responses, they do not depress lymph node cell responses from MDP or saline-treated mice.

The very interesting results of these studies need to be expanded in the future, during this contract.

II. Studies on the mechanism of protection (section 2, beginning p. 9 of our proposal) led to some unexpected and very interesting results. In the course of studies on macrophage activation for killing _B. dermatitidis_, we were exploring further a method of activation which involved immunizing mice by subcutaneous infection, and challenging them with antigen. It had appeared that this method could produce a peritoneal macrophage which could kill this extracellular fungus, a property shared by macrophages from animals stimulated in vivo with conA or with BCG (Brummer et al, Infect. Immun. 39:817-822, 1983), but not by normal resident macrophages, nor by polymorphonuclear leukocytes (PMN) (which actually enhance _B. dermatitidis_ growth). Comparisons of these different methods of activation was an objective of this line of inquiry. It was of interest then that this immunologically activated macrophage-containing

population appeared to be affected by the presence of antibody and complement, a property not shared by macrophages activated by con A and BCG, and the duration of the killing ability was more short-lived. This led us to examine the possibility that the PMN in the immunologically-activated peritoneal exudate might be different than the impotent (non-fungicidal) PMN induced by inflammatory agents. This examination uncovered some data of broad interest, in that it implies that the PMN can be activated (as demonstrated by fungal killing) by immunological stimuli, a concept with little precedent in the literature (which tends to consider the PMN an all-or-nothing cell). This has important implications for immunomodulators, and their mechanisms of action. This work was presented to the annual meeting of the Reticuloendothelial Society. A full paper has also been submitted for publication.

These findings can be summarized as follows: the interaction of elicited murine polymorphonuclear (PMN) neutrophils and the thermally dimorphic fungal pathogen B. dermatitidis in vitro was studied. PMN elicited intraperitoneally with thioglycollate, in normal mice or mice immune to B. dermatitidis, failed to reduce colony forming units (CFU) of B. dermatitidis in the inoculum in a 4 hr vitro assay, even in the presence of 10% fresh immune serum. In contrast, PMN elicited intraperitoneally in immune mice by injection of non-viable B. dermatitidis cells significantly reduced inoculum CFU (60 ± 5%) under the same conditions. Furthermore, non-viable B. dermatitidis i.p. in normal mice or non-viable Candida albicans i.p. in immune mice failed to elicit peritoneal exudate cells that reduced inoculum CFU in this system. These results support the concept that PMN, elicited in a site by means of an immunological reaction, acquired enhanced microbicidal activity. The fungicidal activity of immunologically elicited PMN was shown to be most effective at higher effector to target cell ratios (1000:1), maximal within two hours of co-culture, and significantly enhanced in the presence of fresh immune serum compared to heat-in activated immune serum, normal mouse serum, or fetal bovine serum. Such PMN also had significantly enhanced fungicidal activity against C. albicans compared to normal PMN. Fungicidal activity was abrogated in the presence of catalase, implicating hydrogen peroxide generation as the killing mechanism in the activated cells.

Activation of PMN in this manner was then also shown to produce a broadly fungicidal cell, which could kill another fungus previously thought to be resistant to PMN killing. The purpose of this study was to test the fungicidal activity of thioglycollate elicited PMN from normal mice and immunologically activated murine PMN against endospores and arthroconidia of Coccidioides immitis. Activated PMN were obtained from peritoneal exudates of B. dermatitidis immunized BALB/cByJ mice 24 hrs after giving nonviable B. dermatitidis cells i.p. Density gradient centrifugation of peritoneal exudate cells on metrizamide gradients (14.5% over 15.5%) at 1500 rpm for 20 min. produced a pellet of cells enriched for PMN and reduced in monocyte-macrophages. Such activated PMN were previously reported to kill B. dermatitidis and to have enhanced candidacidal activity. Using a similar in vitro assay system where high effector to target cell ratios (500:1) were employed, endospores were killed (83 ± 2.8%) only by activated PMN. Although activated PMN also had enhanced candidacidal (75.5 ± 4.9% vs 44 ± 5.6%) activity, they failed to kill arthroconidia. These results indicate that C. immitis endospores, but not arthroconidia, are susceptible to the elevated fungicidal mechanisms of activated PMN, which we have previously reported to be inhibited by catalase. These results also suggest that activated PMN in C. immitis immunized mice could play a role in protection against this pathogen in its parasitic phase.

Further work showed that we could reproduce immunological activation of PMN with lymphokines. This now enables dissection in vitro of the afferent limb of what is likely in vivo an important mechanism, i.e., communication of cell-mediated immunity with the inflammatory response. Study of activation of PMN is thus freed of the complexities of the in vivo system of activation described above.

As described above, PMN elicited in B. dermatitidis immune mice with nonviable B. dermatitidis cells i.p. acquired ability to kill B. dermatitidis (60 ± 5%) and exhibited enhanced candidacidal activity. Spleen cells from mice immunized with B. dermatitidis were cultured ($10\text{-}20 \times 10^6$/ml) with nonviable B. dermatitidis cells (0-500 ug/ml) for increasing periods (2, 4, 6 days). Supernatants were tested for activity on PMN from normal mice elicited with thioglycollate (1 ml, i.p., 4 hours, ≥ 85% PMN). Only supernatants from cultures stimulated with 200 to 500 µg of B. dermatitidis for 4 to 6 days activated PMN for killing B. dermatitidis. (31 ± 6%). Generation of activity was antigen specific, in that supernatants from cultures stimulated with heat killed C. albicans did not have significant activity. Supernatants from normal spleen cells stimulated with con A also exhibited ability to activate PMN for killing of B. dermatitidis (29 ± 3%). These results demonstrate that supernatants generated by antigen or mitogen stimulation of spleen cells mediate the acquisition of ability to kill B. dermatitidis by PMN in vitro and provide evidence that lymphokines are involved in the in vivo activation of PMN for enhanced fungicidal activity.

Using the Blastomyces system, with a baseline of zero killing by unstimulated PMN, we have an excellent basis for studying enhancement by immunomodulating drugs.

III. The other line of attack in our proposal is the study of other immunomodulating agents, and their effects on macrophages in antimicrobial functions, using fungi as the target cells. The goals are described in the proposal, but include the exploration of different classes of immunomodulators for possible clinical applications, making comparative studies of effect and mechanism possible, and the possible use of combinations of agents with complementary and/or synergistic effects in vivo.

The first of these proposed was CP-46665, a synthetic immunomodulator with several promising features. The drug is more active given in a lipid diluent, which presumably enhances drug delivery to the reticuloendothelial system. This is of interest because of extensive current work using liposomes to enhance drug delivery to their sites of action.

In summary, our first round of experiments indicates CP-46665 does enhance antimicrobial activity, using C. albicans as the target, 2 different types of professional phagocytes, and killing as the parameter measured.

The table below summarizes this data. I have omitted the data with the saline controls for brevity, as they appeared indistinguishable from the intralipid controls. Each point represents the mean of 4 wells. "Polymorphonuclear leukocytes" refers to thioglycolate-elicited peritoneal exudate cells (85% PMN). Each data point is the mean of 4 wells, from pooled animals (Balb/cByJIMR).

Table 11

Polymorphonuclear Leukocytes (C. albicans target)

| Group | Day 1 | 2 | 3 | 4 | 6 | 7 |
|---|---|---|---|---|---|---|
| Intralipid | 28 | 56 | 50 | 32 | 38 | 37 |
| CP-0.5 | 31 | 48 | 68 | 43 | 33 | 44 |
| CP-1.0 | 47 | 63 | 53 | 43 | 40 | 47 |
| CP-5.0 | 41 | -- | 56 | 37 | 40 | 37 |
| CP-10.0 | 41 | 37 | 60 | 46 | 40 | 60 |
| No cell control | < 0 | < 0 | 4 | 0 | 2 | < 0 |

Particularly noteworthy results are underlined. Some of the increases are really quite remarkable, e. g., day 1 result with 1.0 represents a 68% boost in killing over intralipid alone, 10.0 on day 4 a 44% boost and on day 7 a 62% boost. I'm not sure I see a clear preferred dose or interval.

Table 12

Alveolar Macrophages (C. albicans target)

| Group | Day 1 | 2 | 3 | 4 | 6 | 7 |
|---|---|---|---|---|---|---|
| Intralipid | 35 | 15 | 45 | 28 | 42 | 42 |
| CP-0.5 | 33 | 34 | 25 | 38 | 33 | 47 |
| CP-1.0 | 48 | 20 | 48 | 41 | 45 | 41 |
| CP-5.0 | 57 | 49 | 37 | 53 | 50 | 48 |
| CP-10.0 | 34 | 22 | 35 | 49 | 36 | 58 |
| No cell control | 2 | <0 | <0 | <0 | <0 | <0 |

Again, some of the increases are impressive; e. g., the underlined result on day 1 represents a 69% boost, day 2 a 226% boost, day 4 an 89% boost, and day 7 a 38% boost. The trend seems a bit clearer here.

We then proceeded to look further at selected doses based on the above, and to zone in on the first couple of days after administration.

Table 13

Alveolar macrophages, day 1

| Group | Percent kill | Percent reduction from intralipid |
|---|---|---|
| Saline control | <0 | -- |
| Intralipid alone | 10 | -- |
| CP 1.0 | 24 | 16 |
| CO 5.0 | 43 | 37 |

Table 14

PMN, day 1

| Group | Percent kill | Percent reduction from intralipid |
|---|---|---|
| No cells | <0 | -- |
| Saline control | 45 | -- |
| Intralipid alone | 45 | -- |
| CP 1.0 | 50 | 9 |
| CP 5.0 | 53 | 14 |

As indicated, the story looks a bit clearer with alveolar macrophages than PMN.

Table 15

Alveolar macrophages, day 1 after CP 46665

| | Percent kill | Percent reduction from intralipid |
|---|---|---|
| Serum alone (no cells) | 10 | -- |
| Intralipid (IL) in vivo and cells | 17 | -- |
| CP 5.0 and cells | 47 | 37 |

Table 16

Alveolar macrophages, day 2 after CP 46665

| | Percent kill | Percent reduction from intralipid |
|---|---|---|
| Serum alone | <0 | -- |
| IL in vivo and cells | 4 | -- |
| CP 5.0 and cells | 16 | 11 |

Even though there was some die-off in the control, the boosting effect of CP 5.0 in vivo on alveolar macrophages appears consistent. The effect seems less 1 day later.

We then looked at whether there was a direct effect of the drugs on C. albicans or on PMN when added in vitro.

Table 17

Effect of reagents in vitro and on PMN in vitro

| | Percent kill | Percent reduction from intralipid |
|---|---|---|
| Serum Alone | 6 | Not calculated |
| IL and serum | <0 | " |
| CP 5 mcg/ml in IL and serum | <0 | " |
| CP 50 mcg/ml in IL and serum | 4 | " |
| PMN + serum | 50 | " |
| PMN + IL and serum | 9 | " |
| PMN, CP 5 mcg/ml in IL and serum | 24 | " |
| PMN, CP 50 mcg/ml in IL and serum | 9 | " |

IL or CP appears to have no direct effect in killing Candida in the concentration tested. However, IL seems to interfere with PMN killing. Therefore, the direct effect of CP on PMN can't be evaluated. We repeated these experiments not using IL as the vehicle for CP in vitro, and used medium (on the assumption CP would be soluble) for the vehicle and control.

This time the CP 46665 (CP) was incubated with the cells for 1 hr prior to challenge with C. albicans.

Table 18

| | % reduction of inoculum CFU |
|---|---|
| Serum | <0 |
| PMN + serum | 59 |
| CP 5 mcg/ml + PMN + serum | 60 |
| CP 50 mcg/ml + PMN + serum | 43 |

Conclusion: CP has no direct effect on PMN killing of Candida.

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IV. Because of (a) the interest in using interferon in clinical immunotherapy, (b) reports that interferon enhances phagocytosis and killing of bacteria (possibly by an action of superoxide dismutase), and (c) the availability of highly potent, purified preparations of interferon resulting from recombinant DNA techniques (cloning the interferon gene into bacteria), we have initiated experiments to study possible effects of interferon on macrophage antimicrobial activity, using fungi as the targets.

Initial experiements have indicated that human alpha interferon in doses of 10-1000 units/ml (corresponding to levels achievable in human serum after IV administration) (a) does not enhance phagocytosis of C. albicans by human PMN, (b) does not enhance killing of C. albicans by human PMN, (c) does not enhance killing of C. albicans by human monocytes after overnight incubation. Further studies are in progress.

We first showed a lack of effect of interferon (IF) itself.

Table 19

Direct effect of IF on Candida (% kill of inoculum)

| Units IF/ml | Expt. 1 | Expt. 2 |
|---|---|---|
| 0 | 0 | 7 |
| 10 | 1 | ND* |
| 100 | <0 | ND |
| 1000 | <0 | 5 |

*Not done.

Conclusion: No direct effect of IF on Candida. Note fungus only exposed to IF for 2 hrs. (controls for experiments below).

Subsequent experiments revealed no effect of IF at 1000 u/ml in a 24-48 hour standard MIC assay.

Effect of IF on phagocytosis of Candida by human polymorphonuclear leukocytes

(PMN) was studied. One hour co-cultivation, 1:3 cell to target ratio, IF mixed with PMN at start of assay. A cytocentrifuge preparation and Geimsa stain method were used.

Table 20

| U. IF/ml | Percent PMN with ingested Candida |
|---|---|
| 0 | 28.5 |
| 10 | 27.5 |
| 100 | 28.0 |
| 1000 | 31.5 |

Conclusion: No significant effect on PMN phagocytosis.

The effect of IF on phagocytosis of Candida by human monocytes was studied. IF was incubated with monocytes overnight, which were then washed. A 1:3 monolayer cell to target ratio, 2 hr co-cultivation, and a subtraction method were used to determine phagocytosis.

Table 21

| U. IF/ml | Percent Candida phagocytosed |
|---|---|
| 0 | 47 |
| 10 | 55 |
| 100 | 55 |
| 1000 | 55 |

Conclusion: No significant effect on monocyte phagocytosis.

The effect of IF on killing of Candida by PMN in a 500:1 cell to target ratio was studied, using a tumbling suspension. IF was mixed with PMN at start of assay, 2 hr co-cultivation.

Table 22
Effect of IF on killing by PMN

| | % reduction of inoculum CFU | |
|---|---|---|
| U. IF/ml | Expt. 1 | Expt. 2 |
| 0 | 56 | 73 |
| 10 | 41 | 69 |
| 100 | 42 | 58 |
| 1000 | 18 | 49 |

Conclusion: Decreased killing by PMN in presence of IF.

The effect of IF on killing of Candida by monocytes was studied using a 500:1 monolayer cell to target ratio. IF incubated with cells overnight then washed, 2 hr co-cultivation.

Table 23
Effect of IF on killing by monocytes

| | % reduction of inoculum CFU | |
|---|---|---|
| U. IF/ml | Expt. 1 | Expt. 2 |
| 0 | 24 | 31 |
| 10 | 26 | 30 |
| 100 | 27 | 25 |
| 1000 | 14 | 12 |
| 0 (and no monocytes) | <0 | <0 |

Conclusion: Decreased killing by monocytes in presence of IF.

V. As a representative of another class of immunomodulating agents, we have performed some studies of the effect of a thymosin preparation on cell-mediated immune responses. "Thymosin" is used as a term to encompass a family of molecular species, probably inter-related, whose origin is the thymus gland and whose functions are complex, but appear principally to involve action on T lymphocytes. These actions relate to particular T cell subsets, and affect maturation and probably expression of function and/or receptors. Our preliminary studies concern a circulating member of this family, found in the prealbumin fraction of human serum. Recent studies in other labs have isolated a single molecule in this fraction, which appears to have the properities of the crude fraction, and it has been synthesized. In our preliminary in vitro studies with patients with granulomatous diseases and healthy controls, we find (a) that this preparation affects in vitro lymphocyte proliferative responses to antigen and mitogen, but not baseline tritiated thymidine incorporation, (b) boosting of responses 75-85% of the time but depression 15-25% of the time, (c) more impressive results with patients than with healthy controls, implying an ability to restore toward normal rather than "boost", (d) a dose of 1000 mcg/ml appears optimal, (e) effects in different granulomatous diseases are strikingly divergent, e. g., our results indicate that response of patients with coccidioidomycosis to coccidioidin are boosted, whereas responses of tuberculosis patients to tuberculin are depressed, implying different regulatory mechanisms in clinically similar diseases. Further studies are required.

In more detail, prealbumin fraction of human serum which apparently contains a circulating form of the hormone, thymosin (thy), was studied in vitro with the lymphocytes of healthy subjects and patients with coccidioidomycosis and tuberculosis. Lymphocyte blastogenesis in response to a mitogen (phytohemagglutinin) and specific antigen (spherulin and PPD, respectively) were studied in the presence and absence of thy. Peripheral blood cells were fractionated by differential centrifugation, and mononuclear cells assayed. Initial studies indicated that thy alone does not stimulate blastogenesis. Initial dose-response studies indicated a concentration of 1 mg/ml in cultures was most consistently optimal in boosting reactivity of coccidioidomycosis patients to mitogen or antigen, but the effect to be described on tuberculosis patients occurred over a broad dilution range. Changes of >50% are considered significant. Six of 8 coccidioidomycosis patients responses to spherulin were boosted, 1 was depressed, and 1 not significantly affected. Two of 2 skin test positive coccidioidomycosis patients were boosted, and 4 of 6 skin test negative. Responses to mitogen were less impressively affected; 3 boosted, 1 depressed, and 4 not affected. Two of 3 coccidioidal skin test positive controls were boosted in their antigen response. In contrast, thy depressed tuberculosis patient response to antigen (4 of 5, one not affected). The mitogen response was depressed in 1 and unaffected in 4. In vitro response to PPD was unaffected in most PPD skin test positive controls. Overall, 4 of 15 controls had boosted mitogen responses. Low nonspecific responses to PPD or spherulin were further depressed in all skin test negative controls. These results indicate thy generally results in boosting of in vitro responses of coccidioidomycosis patients to antigen or mitogen, but depresses responses in tuberculosis patients. This suggests that peripheral blood mononuclear subpopulations that differ in their dominance in the two granulomatous diseases may be affected in different directions by thy.

For the second and third years of this contract, we plan to follow the plan originally given in detail in our proposal, as well as exploiting new findings as described above. Briefly, these will include (a) further definition of the cell subsets affected by MDP, (b) determining the mechanism of MDP protection against Blastomyces infection in vivo, by assaying humoral immunity and cell-mediated immunity (including macrophage) function in our model after in vivo MDP administration, (c) sharpening the picture of the immunological profile of the newer immunomodulators preliminarily studied.

Publications from this contract: (1) "Positive immunological modulation of murine polymorphonuclear neutrophils for fungicidal activity against Blastomyces dermatitidis", E. Brummer, A.M. Sugar, D.A. Stevens; J. Reticuloendothelial Soc. (RES) 34:157-158, 1983. (2) "Immunological activation of polymorphonuclear neutrophils for fungal killing: studies with murine cells and Blastomyces dermatitidis in vitro", E. Brummer, A.M. Sugar, D.A. Stevens; submitted for publication.

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